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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

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Applicant's or agent's file reference Case 21408	FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/PEA/416)	
International application No. PCT/EP 03/10473	International filing date (day/month/year) 19.09.2003	Priority date (day/month/year) 23.09.2002
International Patent Classification (IPC) or both national classification and IPC C12N15/53		
Applicant DSM IP ASSETS B.V. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 8 sheets, including this cover sheet.
- ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the opinion
- II ☐ Priority
- III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

Date of submission of the demand 11.03.2004	Date of completion of this report 20.01.2005
Name and mailing address of the international preliminary examining authority:  European Patent Office - P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk - Pays Bas Tel. +31 70 340 - 2040 Tx: 31 651 epo nl Fax: +31 70 340 - 3016	Authorized Officer Madruga, J Telephone No. +31 70 340-3121 

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. **PCT/EP 03/10473**

1. Basis of the report

1. With regard to the **elements** of the international application (*Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17)*):

Description, Pages

1-12 as originally filed

Sequence listings part of the description, Pages

1-14 received on 16.01.2004 with letter of 15.01.2004

Claims, Numbers

1-8 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
☐ the language of publication of the international application (under Rule 48.3(b)).
☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☒ contained in the international application in written form.
☐ filed together with the international application in computer readable form.
☒ furnished subsequently to this Authority in written form.
☒ furnished subsequently to this Authority in computer readable form.
☒ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

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5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)).

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes: Claims	1-5
	No: Claims	6-8
Inventive step (IS)	Yes: Claims	
	No: Claims	1-8
Industrial applicability (IA)	Yes: Claims	1-8
	No: Claims	

2. Citations and explanations

see separate sheet

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following documents:

- D1: WANNER PETER ET AL: 'Purification and characterization of two enone reductases from *Saccharomyces cerevisiae*' EUROPEAN JOURNAL OF BIOCHEMISTRY, BERLIN, DE, vol. 255, no. 1, July 1998 (1998-07), pages 271-278, XP002202649 ISSN: 0014-2956
- D2: MIRANDA MANUEL ET AL: 'Nucleotide sequence and chromosomal localization of the gene encoding the old yellow enzyme from *Kluyveromyces lactis*' YEAST, vol. 11, no. 5, 1995, pages 459-465, XP008028593 ISSN: 0749-503X
- D3: EP-A-1 236 796 (DAICEL CHEM) 4 September 2002 (2002-09-04)
- D4: WO 03 070924 A (ROCHE VITAMINS AG ;KATAOKA MICHIIHIKO (JP); SHIMIZU SAKAYU (JP)) 28 August 2003 (2003-08-28)
- D5: WO 03 070959 A (ROCHE VITAMINS AG ;WADA MASARU (JP); SHIMIZU SAKAYU (JP)) 28 August 2003 (2003-08-28)
- D6: KATAOKA M ET AL: 'OLD YELLOW ENZYME FROM CANDIDA MACEDONIENSIS CATALYZES THE STEREOSPECIFIC REDUCTION OF THE C = C BOND OF KETOISOPHORONE' BIOSCIENCE, BIOTECHNOLOGY AND BIOCHEMISTRY, XX, XX, vol. 66, no. 12, December 2002 (2002-12), pages 2651-2657, XP009011699 ISSN: 0916-8451

The present application concerns the isolation of a DNA comprising a nucleotide sequence encoding for a protein having enone reductase activity from *Candida Kefyr* (*C. macedoninsis*) and the production of levodione from ketoisophorone using said enzyme.

1 NOVELTY (Art. 33(2) PCT)

- 1.1 D1 discloses an enzyme from *Saccharomyces cerevisiae* (EII) which has enone reductase activity, a molecular weight of 64 kDa, uses NADH as co-factor, is specific for α and β -unsaturated ketons and has an optimal pH of 4.8 (D1, pag. 273, right-hand column, paragraph 1 to page 275, right-hand column, paragraph 1, figures and tables). The enone reductase (EII) disclosed in D1 is shown to use

ketoisophorone (2,6,6-trimethyl-2-cyclohexen-1,4-dione) as substrate and that levodione ((6R)-2,6,6-trimethyl-1,4-cyclohexandione) was produced (D1, table 3, page 275, right-hand column, paragraph 1, page 277, right-hand column, paragraphs 1,2).

- 1.2 The optimum values referred to in claim 1 (e.g. for temperature and pH) are **relative values** which depend on the conditions used in the assay, and as such are not sufficient to distinguish the claimed subject-matter from the available prior art. D1 discloses that EII has an optimum temperature of 30-38 °C but D1 is silent about the pH used; it cannot be excluded that under certain conditions the enzyme disclosed in D1 has an optimal temperature of 55-60°C at pH 7.4.
- 1.3 Thus, the subject-matter of claim 6 is not new. In addition, the process for the production of levodione in claims 7 and 8 is also not new (Art. 33(2) PCT). The specific conditions referred to in claims 7 and 8 are entirely optional ("...e.g. at pH values...").
- 1.4 D2 discloses a nucleic acid molecule from *Kluyveromyces lactis* which is 79.3 % identical to SEQ ID NO: 1 in a 1198 nucleotides overlap. The protein encoded by said sequence is an "Old Yellow Enzyme NADPH oxidase" and has 80% sequence identity to SEQ ID NO: 2 of the application in a 398 amino acids overlap. The enzyme disclosed in D2 has a molecular mass of 44822 Da. Thus, the enzyme in D2 is different from the subject-matter of present claims.
- 1.5 D3 discloses a nucleic acid sequence encoding for a enone reductase from *Kluyveromyces lactis* which has activity on alpha beta unsaturated ketones, uses NADH and NADPH but has a molecular weight of 40-42 kDa (D3 examples 1-11). Thus, the enzyme in D3 is different from the subject-matter of present claims.
- 1.6 The present application does not satisfy the criterion set forth in Article 33(2) PCT because the subject-matter of claims 6-8 is not new in respect of prior art as defined in the regulations (Rule 64(1)-(3) PCT).

2 INVENTIVE STEP (Art. 33(3) PCT)

- 2.1 Document D1 is considered to represent the most relevant state of the art in respect to inventive step. The subject-matter of claim 1 differs from D1 in that said

claim is directed to the **nucleic acid** encoding for an enone reductase.

2.2 The **problem** to be solved by the present invention may therefore be regarded as providing a further DNA encoding for an enone reductase. The **solution** proposed in claim 1 is a mere recitation of the problem to be solved, namely the recitation of the properties and activity of the encoded enzyme and as such, obvious for a skilled person with knowledge of D1. Thus, the solution proposed in claim 1 cannot be considered as involving an inventive step (Art. 33(3) PCT).

2.3 Dependent claims **2 and 3** do not appear to contain any additional features which, in combination with the features of any claim to which they refer, involve an inventive step for the following reasons:

2.3.1 A skilled person with knowledge of D1 would attempt the cloning of the DNA encoding for the enone reductase (EII), with high expectations of success. Said DNA can be considered as: (a) an allelic variant of SEQ ID NO: 1, (b) a sequence coding for a polypeptide having the amino acid sequence shown in SEQ ID NO: 2, in which one or more amino acids are added, inserted, deleted and/or substituted, but having the enone reductase activity and, (c) a sequence which will hybridize to SEQ ID NO: 1 under stringent hybridisation conditions.

2.3.2 The terms "**allelic variant**" and the expression "...the amino acid sequence shown in SEQ ID NO: 2, in which one **or more** amino acids are **added, inserted, deleted and/or substituted**,..." do not allow a person skilled in the art to determine the precise extension and the technical features referred to by said terms; this terms are thus interpreted in the broadest possible sense, which means that all possible sequences are included.

2.3.3 Thus, the subject-matter of claims **1-3** is not considered inventive (Art. 33(3) PCT).

2.4 Accordingly, claims **4 and 5** are not regarded as inventive since they represent standard techniques which are obvious embodiments for a skilled person.

2.5 The present application does therefore not satisfy the criterion set forth in Article 33(3) PCT and the subject-matter of claims **1-5** does not involve an inventive step (Rule 65(1)(2) PCT).

3 CLARITY (Art. 6 PCT)

- 3.1 Claim 1 does not meet the requirements of Article 6 PCT in that the matter for which protection is sought is not defined. The claim attempts to define the subject-matter in terms of the result to be achieved, namely, the characteristics of an enzyme encoded by the DNA claimed. In this instance, such a formulation is not allowable because it appears possible to define the subject-matter in more concrete terms, i.e. in terms of structural features of the DNA, e.g. its sequence.
- 3.2 The term "**stringent hybridization conditions**" in claim 3 does not have a precise technical meaning. To comply with the clarity requirements of Article 6 PCT, it should be indicated which specific conditions are meant.

4 PRIORITY AND INTERFERING DOCUMENTS

- 4.1 D4 is an international application from the same applicant. D4 discloses an enzyme having all the properties referred to in claim 1 and a process to produce levodione using said enzyme. D4 claims priority from EP02003967, filed on 22 February 2002, and the enzyme, its properties and the process to produce levodione using said enzyme were already disclosed in said priority document. Thus, the subject-matter disclosed in EP02003967 challenges the priority claim of the present application.
- 4.2 D5 is an international application from the same applicant. D5 discloses an enzyme having all the properties referred to in claim 1 and a process to produce levodione using said enzyme. D4 claims priority from EP02003968, filed on 22 February 2002, and the enzyme, its properties and the process to produce levodione using said enzyme were already disclosed in said priority document. Thus, the subject-matter disclosed in EP02003968 challenges the priority claim of the present application.
- 4.3 D6 discloses an "Old Yellow Enzyme" from *Candida macedoniensis*, having all the properties referred to in claim 1 and a process to produce levodione using said enzyme.

- 4.4 Although D4-D6 do not constitute prior art within the meaning of Rule 64.1(b), it appears that these documents disclose all the features of claims referred. D4-D6 might therefore be taken into consideration in the regional phase.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
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(10) International Publication Number
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- (71) Applicant (for all designated States except US): DSM IP ASSETS B.V. [NL/NL]; Het Overloon 1, NL-6411 TE Heerlen (NL).
- (72) Inventors; and
- (75) Inventors/Applicants (for US only): KATAOKA, Michihiko [JP/JP]; 24-308 Okazaki-Irie-cho, Sakyo-ku, Kyoto-shi, Kyoto-fu, Kyoto 606-8322 (JP). SHIMIZU, Sakayu [JP/JP]; 6-9 Yamashita-cho, Tokiwa, Ukyo-ku, Kyoto-shi, Kyoto-fu, Kyoto 616-8212 (JP).
- (74) Agents: KELLER, Günter et al.; Lederer & Keller, Prinzregentenstrasse 16, 80538 München (DE).
- (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- Published:**
- with international search report
 - before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments
- (88) Date of publication of the international search report: 27 May 2004
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

(54) Title: ENONE REDUCTASE GENE AND MICROBIAL PRODUCTION OF LEVODIONE

(57) Abstract: Disclosed is an isolated DNA comprising a nucleotide sequence coding for an enzyme having enone reductase activity wherein the enzyme is characterized by the following physico-chemical properties: (a) molecular mass: 61,300 ± 5,000 Da (estimated using gel filtration, consisting of one subunit); (b) co-factor: NADPH and NADH; (c) substrate specificity: active on α,β-unsaturated ketons; (d) optimum temperature: 55-60°C at pH 7.4; and (e) optimum pH: pH 4.5-8.5.

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INTERNATIONAL SEARCH REPORT

International Application No.

PCT/EP 03/10473

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/53 C12N9/02 C12P7/26

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12P C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EPO-Internal, SEQUENCE SEARCH, WPI Data, PAJ, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WANNER PETER ET AL: "Purification and characterization of two enone reductases from <i>Saccharomyces cerevisiae</i> " EUROPEAN JOURNAL OF BIOCHEMISTRY, BERLIN, DE, vol. 255, no. 1, July 1998 (1998-07), pages 271-278, XP002202649 ISSN: 0014-2956 the whole document	1-8
P, X, L	WO 03 070924 A (ROCHE VITAMINS AG ;KATAOKA MICHIIHIKO (JP); SHIMIZU SAKAYU (JP)) 28 August 2003 (2003-08-28) L: PRIORITY the whole document	6-8

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- *&* document member of the same patent family

Date of the actual completion of the international search

12 March 2004

Date of mailing of the international search report

23/03/2004

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INTERNATIONAL SEARCH REPORT

International Publication No.

PCT/EP 03/10473

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	WO 03 070959 A (ROCHE VITAMINS AG ; WADA MASARU (JP); SHIMIZU SAKAYU (JP)) 28 August 2003 (2003-08-28) the whole document	6-8
P, X	----- KATAOKA M ET AL: "OLD YELLOW ENZYME FROM CANDIDA MACEDONIENSIS CATALYZES THE STEREOSPECIFIC REDUCTION OF THE C = C BOND OF KETOISOPHORONE" BIOSCIENCE, BIOTECHNOLOGY AND BIOCHEMISTRY, XX, XX, vol. 66, no. 12, December 2002 (2002-12), pages 2651-2657, XP009011699 ISSN: 0916-8451 the whole document	6-8
A	----- MIRANDA MANUEL ET AL: "Nucleotide sequence and chromosomal localization of the gene encoding the old yellow enzyme from Kluyveromyces lactis" YEAST, vol. 11, no. 5, 1995, pages 459-465, XP008028593 ISSN: 0749-503X the whole document	
A	----- EP 1 236 796 A (DAICEL CHEM) 4 September 2002 (2002-09-04) the whole document	
A	----- STOTT KELVIN ET AL: "Old Yellow Enzyme: The discovery of multiple isozymes and a family of related proteins" JOURNAL OF BIOLOGICAL CHEMISTRY, AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, US, vol. 268, no. 9, 25 March 1993 (1993-03-25), pages 6097-6106, XP002252405 ISSN: 0021-9258 the whole document	
A	----- US 6 428 991 B1 (FUKUOKA MASATSUKA ET AL) 6 August 2002 (2002-08-06) the whole document	
A	----- US 4 072 715 A (BOGUTH WALTER ET AL) 7 February 1978 (1978-02-07) cited in the application the whole document	

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 03/10473

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 03070924	A	28-08-2003	WO 03070924 A1	28-08-2003
WO 03070959	A	28-08-2003	WO 03070959 A2	28-08-2003
EP 1236796	A	04-09-2002	JP 2002247987 A	03-09-2002
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			GB 1508196 A	19-04-1978